Peptidolytic enzymes in different larval stadium of housefly *Musca domestica*

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ABSTRACT: Four classes of peptidolytic enzymes were described in insects. Many authors have found predominant activity belonging to trypsin-like and chymotrypsin-like activity. By the use specific chromogenic substrates and hemoglobin we have determined enzyme activity in three stages of larval development of housefly. In contrast to above mentioned data we have found, that major part of peptidolytic activity in this insect is of aminopeptidase nature. Other observed peptidolytic activity formed only minority part. Apparently the highest activities to all examined substrates were found in first larval stadium of housefly. Inhibitory studies by class specific inhibitors and influence of metal ions and chelating agent on enzyme activity have shown, that aminopeptidase-like enzymes belong to metalloproteinase group.

Keywords: trypsin; chymotrypsin; aminopeptidases; housefly *Musca domestica*

In animal kingdom, digestion of both food and intracellular proteins too, is accomplished by initial degradation of proteins into peptides by endopeptidases, followed by further degradation into smaller peptides and finally to amino acids by exopeptidases. Both types of peptidolytic enzymes are localized in many subcellular organelles, in cytoplasm and as membrane components, too. As protein degradation is essential physiological process, also in insects, considerable effort was made by many authors to elucidate which types of peptidolytic enzymes are involved in this process. Moreover there are also other reasons for characterization of these enzymes in insects. Larval peptidolytic enzymes and their inhibitors are now involved in development of new strategies and approaches for enhancing of resistance of agricultural crops and forest trees to insect herbivores (Terra and Ferriera, 1994). These enzymes may be involved also in activation and degradation of some insecticidal proteins of bacterial origin (de Maagd et al., 2001) and are presumed to participate in major part in events joined with the use of larvae in clinical practice (Sherman, 2002; Blahovec et al., 2005).

All four classes of digestive proteinases which have been identified in vertebrates also occur in insects (Reeck et al., 1999). Many authors have reported, that in many insects predominant peptidolytic activity belongs to serine proteinases of two different subclasses, trypsin-like and chymotrypsin-like (Johnston et al., 1995; Lee and Anstee, 1995; Lam et al., 1999; Valaitis et al., 1999; Wagner et al., 2002) and to less active aspartyl proteinase and metalloproteinase (Chambers et al., 2003; Wang et al., 2005). Few reports were found on these enzymes in housefly (Lemos and Terra, 1991, 1992).

In the present paper we report on larval peptidolytic enzymes of housefly *Musca domestica*. We describe these enzymes in three different larval stages and compare them. With the use of specific chromogenic substrates and natural substrate denatured hemoglobin, we demonstrated that the major part of peptidolytic activity in all examined larval stages belongs to aminopeptidases and not to trypsin-like, or chymotrypsin-like activity.

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MATERIAL AND METHODS

Enzyme activity was assayed in crude homogenate and supernatant fraction obtained after centrifugation. Enzymes were determined to both, five synthetic chromogenic substrates and natural protein substrate, denatured hemoglobin, too. Chromogenic substrates and specific inhibitors were from Sigma – Aldrich (St.Louis, MO, USA) and Fluka Chemie (Germany).

Substrates: N-Succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide (N-Suc-$(Ala)_2$-Pro-Phe-*p*-NA), N_α-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (DL-BAPA), N_α-Benzoyl-L-arginine *p*-nitroanilide (L-BAPA), N-Succinyl-alanyl-alanyl-alanine *p*-nitroanilide (N-Suc-$(Ala)_3$-*p*-NA), L-leucine *p*-nitroanilide (L-Leu-*p*-NA).

Inhibitors: Trypsin inhibitor from soybean, Phenylmethanesulfonyl fluoride (PMSF), Tosyl-L-phenylalanine chlormethyl ketone (TPCK), Nα-Tosyl-L-lysine chlormethyl ketone (TLCK), Chymostatin, *p*-Chloromercurybenzoic acid, Iodoacetamide.

All other reagents and solvents were of the highest available purity.

Preparation of crude enzyme extracts. Larvae in all larval stages were homogenized with prechilled mortar and pestle in 4 volumes (ml/g) of cold distilled water. Homogenates were centrifuged for 15 min at 6 000 × g. Supernatant was delipidated by chloroform and after centrifugation was used for determination of enzyme activity.

Determination of enzyme activity to chromogenic substrates. Enzymatic activity was determined in homogenate and supernatant fraction of three larval stages of housefly. The reaction rate was estimated by reading of reaction product *p*-nitroaniline at 405 nm. Incubation solution usually contained 640 µl of 100mM Tris/HCl buffer, pH 8.3, 30 µl of enzyme and 30 µl of chromogenic substrate of 10mM concentration dissolved in dimethylformamide (DMF). After incubation at 37°C for 30 min, sometimes 60 min, reaction was stopped by 300 µl of 10% of trichloracetic acid (TCA). Enzyme activity is expressed in U which is defined as enzyme amount which produce 1 µmol *p*-nitroaniline per min per mg of proteins. The values are means of duplicate determinations. Concentration of proteins was determined by Bradford assay with bovine albumin as standard protein (Bradford, 1976).

Determination of enzyme activity to denatured hemoglobin. Urea denatured hemoglobin was prepared by procedure described elsewhere (Blahovec, 1991). When this substrate was used for enzyme determination, incubation time was 2 hours. Enzymatic reaction was stopped by 500 µl of 10% TCA and then 500 µl of reaction solution was alkalized by 5 ml of 100mM NaOH. Amount of enzyme is proportional to released tyrosine residues determined by adding 1.5 ml of Folin reagent and absorbance was measured at 750 nm.

Thermal stability and pH optimum. Influence of temperature on activity of larval enzymes was examined to L-Leu-*p*-NA as substrate. Enzyme extract from first larval stadium was incubated at temperatures from 30°C to 80°C for 20 and 120 min and aliquots were assayed for enzyme determination. For determination of pH optimum, enzyme extract was incubated at 37°C for 30 min with L-Leu-*p*-NA and 2 hours with hemoglobin and L-BAPA as substrates in various buffers with different pH and concentration 100mM. Four groups of buffers were used, Glycine/HCl for pH 2–3, Citrate for pH 4–7, Tris/HCl for pH 8–9.5 and Glycine/NaOH for 10–11.5.

Inhibitor and metal ions studies. Effects of class specific inhibitors of serine, cysteine, aspartic and metallo-proteinases on peptidolytic activity of supernatant fraction of first larval stadium were assayed. Incubation solution contained 750 µl of 100mM Tris/HCl, pH 8.3, 100 µl of enzyme extract and 100 µl of 1mM class specific inhibitor. Mixture was preincubated 10 min at laboratory temperature and 50 µl of L-Leu-*p*-NA was added. After 15 min incubation at 37°C reaction was stopped by 500 µl of 10% TCA and enzyme released *p*-nitroaniline measured at 405 nm. To determine the effects of metal ions and EDTA on enzyme activity, the concentration of added effectors was 10mM.

RESULTS

Distribution of enzymes into different larval stadium and enzyme activity to different substrates

Distribution of enzyme activities into homogenates and supernatant fractions of housefly larvae in three different larval stages is shown in Table 1. All assayed enzymes were present in higher concentration in soluble supernatant fraction. The enzyme activity determined to L-Leu-*p*-NA represented principal peptidolytic activity of both homogenate

Table 1. Peptidolytic activity of different larval stadium of housefly. Enzyme activity was determined to enzyme specific chromogenic substrates and hemoglobin under conditions described in text. Values are means of duplicate determinations

Larval stadium		Enzyme activity in U					
		L-Leu-*p*-NA	DL-BAPA	L-BAPA	Suc-$(Ala)_2$Pro-Phe-*p*-NA	Suc-$(Ala)_3$-*p*-NA	Hemoglobin*
1	Homogenate	40.5	2.20	2.85	2.65	0.35	0.75
	Supernatant	52.0	2.75	3.90	3.25	0.20	0.90
2	Homogenate	12.1	0.75	1.20	0.55	–	–
	Supernatant	14.3	0.85	1.70	0.65	0.20	0.40
3	Homogenate	5.5	–	–	–	0.0	–
	Supernatant	7.5	0.30	0.55	0.40	0.0	0.25

U is defined as enzyme amount which produce 1 μmol *p*-nitroaniline per min per mg of proteins
*U calculated for tyrosine residues

and supernatant fraction of larvae. When compared aminopeptidase-like enzymes (substrate L-Leu-*p*-NA) to so called trypsin- and chymotrypsin-like enzymes (substrates BAPAs and Suc-$(Ala)_2$Pro-*p*-NA) in first larval stadium, the concentration of aminopeptidase-like enzymes, calculated on the base of specific activity, is more than 15 times higher than concentration of trypsin- or chymotrypsin-like enzymes. Low enzymatic activity was observed to denatured hemoglobin and negligible or no activity was found to hydrolysis of Suc-$(Ala)_3$-*p*-NA, substrate used for determination of elastolytic activity. The enzyme activity determined to all types of specific substrates was apparently the highest in first larval stadium and in supernatant fraction specific activity to L-Leu-*p*-NA was approximately 7 times higher than that of third stadium.

Effect of pH and thermal stability

Effect of pH on the activity of larval proteinases and peptidases is illustrated in Figure 1. The enzymes show maximal activity in pH range 8–9 with L-Leu-*p*-NA, L-BAPA and denatured hemoglobin, too as substrates and enzymes are fully inactive below pH 3. With hemoglobin there is second small peak in acidic pH with maximum around pH 4.

As shown in Figure 2 larval peptidases are not very stable with respect to temperature. No loss of activity was observed only bellow 40°C even after a preincubation for 2 hours. However, inactivation was almost total after 2 hours preincubation at 80°C. Some residual activity remained at this temperature after only 20 min of preincubation.

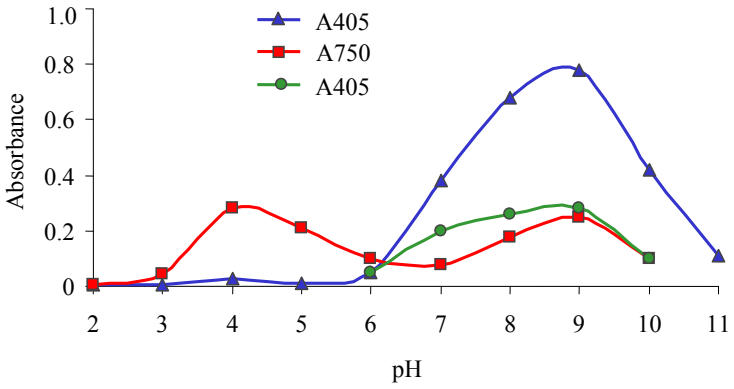

Figure 1. Effect of pH on the activity of larval proteinases and peptidases. The enzyme activity was determined to hemoglobin ■—■, L–BAPA ●—● and L-Leu-*p*-NA ▲—▲ in buffers with different pH under conditions described in text. The following buffers of 100mM concentration were used: Glycine/HCl for pH 2–3, Citrate for pH 4–7, Tris/HCl for pH 8–9.5 and Glycine/NaOH for 10–11.5

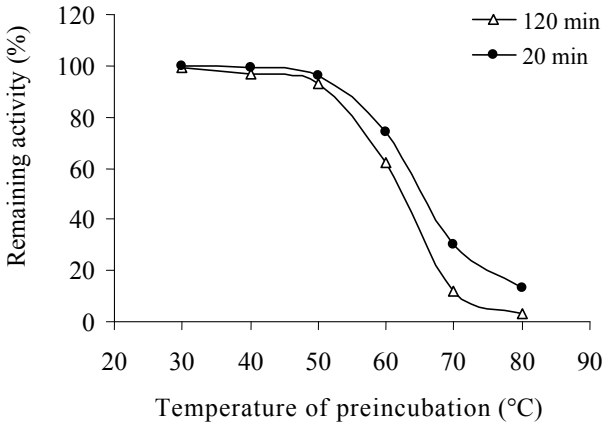

Figure 2. Effect of different temperatures on peptidolytic activity of supernatant fraction of first larval stadium. Enzymes were preincubated at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C for 20 and 120 min and activity was determined to L-Leu-*p*-NA under conditions described in text

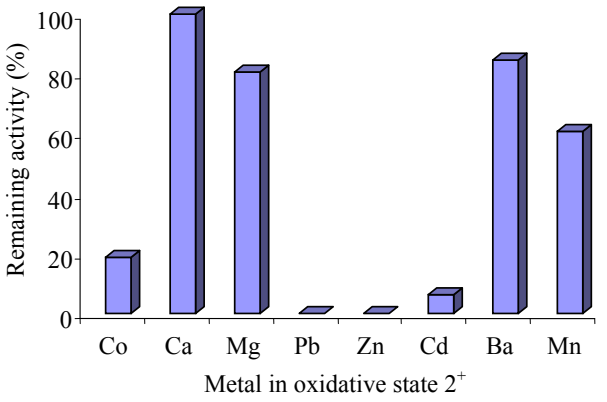

Figure 3. Effect of different metals on enzyme activity of supernatant fraction of first larval stadium. The metals were added to incubation solution in final concentration 1mM and enzyme activity was determined to L-Leu-*p*-NA under conditions described in text

Inhibitors and metal ions effect on enzyme activity

Effect of different metals on enzymes of supernatant fraction of first larval stadium is illustrated in Figure 3. The enzyme activity determined to L-Leu-*p*-NA as substrate was totally inhibited by some bivalent metal ions (Pb, Zn), markedly inhibited by heavy metals (Co, Cd) and Mn reduced activity to about 55%. On the other hand slight inhibition was observed in the presence of Mg and Ba and Ca had no influence on activity. The influence of class specific synthetic inhibitors and natural soybean trypsin inhibitor is shown in Table 2. The enzyme activity was affected neither by serine specific, nor cysteine specific inhibitors. No change of activity was observed also in the presence of natural protein inhibitor from soybean. Enzyme activity was reduced to 60% only by EDTA in 1mM final concentration.

Table 2. Effect of class specific inhibitors, soybean trypsin inhibitor and EDTA on peptidolytic activity of supernatant fraction of first larval stadium. Enzyme activity was determined to L-Leu-*p*-NA under condition described in text

Inhibitor	Inhibition (%)
Iodoacetamide	0
Trypsin inhibitor from soybean	0
N_{α}-Tosyl-L-lysine chloromethyl ketone hydrochloride	6
4-chloro-mercuribenzoic acid	2
Phenyl methanesulfonyl fluoride	0
Tosyl-L-phenylalanine chloromethyl ketone	0
EDTA	62

DISCUSSION

Use of specific chromogenic substrates and denatured hemoglobin enabled us to identify several types of peptidolytic activities in larvae of housefly. In contrast to many other insects (Milne and Kaplan, 1993; Valaitis, 1995; Ortego et al., 1996; Novillo et al., 1999; Wagner et al., 2002) we observed low specific activities for trypsin-like and chymotrypsin-like enzymes, we have found no activity for elastolytic enzymes. The rate of breakdown of natural substrate hemoglobin by non specific proteases, probably cathepsin D-like enzymes (Lemos and Terra, 1991), was also low. On the other hand we have observed unexpectedly high activities in this insect for hydrolysis of L-Leu-*p*-NA, substrate used for determination of aminopeptidases. Specific activity of supernatant fraction of these enzymes in first larval stadium of housefly was at least two order higher, than that reported recently by Wang et al. (2005) for cabbage looper larvae *Trichoplusia ni,* and approximately 20 times higher than observed by Bozic et al. (2003) in *Morimus funereus* larvae. The specific activity of all types

of peptidolytic enzymes apparently decrease with development of larvae and for all enzymes is the lowest in third larval stadium.

Influence of pH on peptidolytic activity of housefly larvae enzymes is similar to many other insect proteinases. Most serine and metallo enzymes of larval origin have pH optimum in slightly alkalic region, pH 8–9 (Lemos and Terra, 1992; Johnson et al., 1995; Lam et al., 1999; Novillo et al., 1999; Bozic et al., 2003). This pH was observed also in our case for hydrolysis of both chromogenic substrates and hemoglobin, too. Natural protein substrate hemoglobin was hydrolyzed also in acidic pH with optimum around pH 4. Activity at this pH however represented only minor part and hydrolysis of both chromogenic substrates at this pH did not occur. With respect to pH optimum and used substrate, this activity is similar to so called cathepsin D-like activity (Lemos and Terra, 1991). Temperature behavior of peptidolytic enzymes of housefly is also similar to many other insect enzymes. The enzymes are relatively little stable and even short 20 minutes preincubation at 60°C has led to apparent loss of activity do to the thermal denaturation of enzyme molecules.

Enzyme activity was affected neither by serine specific, nor cysteine specific inhibitors. No change of activity was observed also in the presence of natural protein inhibitor from soybean. Enzyme activity was reduced to 60% only by EDTA in 1mM final concentration. Similar but not identical results were recently reported by Bozic et al. (2003) for larvae of *Morimus funereus*. They have found, that partially purified aminopeptidases were also strongly ihibited by Cd^{2+}, $Zn^{2+}$and Co^{2+}, but the effect of EDTA in our case was not so pronounced. In contrast to their enzymes we have found no inhibition even by higher ammonium sulfate concentrations (data are not presented).

In conclusion, our results on inhibitory effects of some metals, effects of pH and class specific enzyme inhibitors and substrate specificity clearly indicate, that prevalent amounts of peptidolytic enzymes present in first larval stadium of housefly are of aminopeptidase nature belonging to metalloproteinase group.

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